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<b>(21) International Application Number:</b> PCT/FI91/00239 <b>(22) International Filing Date:</b> 8 August 1991 (08.08.91)  <b>(30) Priority data:</b> 904124 20 August 1990 (20.08.90) FI  <b>(71) Applicant (for all designated States except US):</b> OY ALKO AB [FI/FI]; Salmisaarenranta 7 H, SF-00180 Helsinki (FI).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> ROSSI, Marianne [FI/FI]; Pohjolaantie 16 B 2, SF-04230 Kerava (FI). LINKO, Yu-Yen [FI/FI]; LINKO, Pekka [FI/FI]; Otakallio 2 B 16, SF-02150 Espoo (FI). VAARA, Timo [FI/FI]; Itäinen Puistotie 16 C 14, SF-00140 Helsinki (FI). TURUNEN, Marja [FI/FI]; Raisioitie 7 A 3, SF-00280 Helsinki (FI).		<b>(74) Agent:</b> PAPULA REIN LAHTELA OY; Box 981, SF-00101 Helsinki (FI).  <b>(81) Designated States:</b> AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CI (OAPI patent), CM (OAPI patent), CS, DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GN (OAPI patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MN, MR (OAPI patent), MW, NL, NL (European patent), NO, PL, RO, SD, SE, SE (European patent), SN (OAPI patent), SU <sup>+</sup> , TD (OAPI patent), TG (OAPI patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> OLIGOSACCHARIDE MIXTURE, AND PROCEDURE FOR ITS MANUFACTURING  <b>(57) Abstract</b>  A procedure for manufacturing an oligosaccharide mixture, in which cyclomaltodextrin-glucanotransferase (CGTase; E.C. 2.4.1.19) acts on starch in the presence of acceptor sugar (trehalose or cellobiose), whereby oligosaccharides containing trehalose and cellobiose, and unique of their sugar composition, are produced.		

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# OLIGOSACCHARIDE MIXTURE, AND PROCEDURE FOR ITS MANUFACTURING

The present invention concerns a starch-based  
5 oligosaccharide mixture containing acceptor sugar, and  
a procedure for manufacturing same.

Oligosaccharides containing trehalose and cel-  
lobiose are linear molecules in which one or several  
glucose units are adjoined to acceptor sugar (trehalose  
10 or cellobiose). In the procedure of the invention,  
cyclomaltodextrin-glucanotransferase (CGTase is made to  
act on starch in the presence of acceptor sugar (treha-  
lose or cellobiose), whereby oligosaccharide mixtures  
containing trehalose and cellobiose, with unique sugar  
15 composition, are formed. Under effect of CGTase on  
starch alone, cyclodextrins with ring structure are  
formed, which are not produced in the manufacturing  
procedure on which the invention reads.

Oligosaccharides can be applied as new raw  
20 materials in the foodstuff, animal feed and medical  
industry, and in chemical industry. To this purpose  
certain oligosaccharides have already been launched on  
the market, e.g. starch-based products such as maltose,  
maltotriose, maltotetraose, isomaltose, panose, etc.  
25 Furthermore, lactose-based oligosaccharides and sugar  
alcohols have been produced, such as maltitol, and sac-  
charose-based products, such as 'coupling sugar', fruc-  
tooligosaccharides, palatinose, etc., which have been  
reported in: Alternative sweeteners (1986), edited by  
30 L.O. Nabors and R.C. Gelard, Marcel Deccker Inc., New  
York, U.S.A., p. 165-244 and 309-323, and in: Develop-  
ments in sweeteners - 2 (1983), edited by K.J. Parker  
and M.G. Lindley, Applied Science Publishers Ltd.,  
Essex, England, p. 1-88.

35 The significance of oligosaccharides is based  
on the fact that they are often low in calories, their  
sweetness is milder than that of saccharose, and they

are either less cariogenic than saccharose or not cariogenic at all. In addition to these properties, oligosaccharides have also good technical characteristics and positive effects both physiologically and in view  
5 of health.

A procedure for manufacturing fructooligosaccharides has been disclosed e.g. in the British Patent No. 2,179,946, in which fructosyltransferase is made to act on saccharose. Palatinose, or isomaltulose,  
10 is produced from saccharose with glucosyltransferase, lactose-based oligosaccharides such as 6-galactosyl lactose are produced from lactose with  $\beta$ -galactosidase. In a paper by K. Ajisaka and H. Fujimoto (1989), American Society Meeting, Sept. 10-15, Miami Beach, a procedure for manufacturing trisaccharide containing trehalose, from glucose and trehalose with R. niveus gluco-  
15 amylase, has been disclosed.

As has been reported in the Japanese Patents No. 72 20,373, 75 63,189 and 75 88,290 and in a paper  
20 by Hans Bender (1977), Arch. Microbiol., 111, 271-282, the enzyme used in the present work, or CGTase, is produced both by bacteria of genus Bacillus, such as Bacillus macerans, Bacillus megaterium, Bacillus circulans, Bacillus polymyxa and Bacillus stearothermophilus, and by certain bacteria of genus Klebsiella.  
25

The manufacturing of oligosaccharides with the CGTase employed in the invention has been described in the German Patent No. 2 162,276 and in the British Patent No. 2,019,406, in both of which the enzyme is  
30 made to act on starch or dextrin in the presence of either saccharose or fructose, whereby as product are formed oligosaccharides of 'coupling sugar' type. In the Japanese Patent No. 75 123,832 and in a paper by S. Kitahata and S. Okada (1976), J. Biochem., 79, 641-648,  
35 is disclosed the manufacturing from dextrin or starch, with CGTase, of oligosaccharides containing xylose and sorbose. Determination of the acceptor specificity of

CGTase from various acceptor sugars and from cyclodextrin is described in scientific magazines (D. French et al. (1948), J. Am. Chem. Soc., 70, 3145; D. French et al. (1954), J. Am. Chem. Soc., 76, 2387-2390; S. Kitahata (1982), Kagaku to Kogyo, 56, 127-130). French et al. (1948 and 1954) used in their experiments cyclodextrin for substrate and e.g. cellobiose for acceptor.

In the procedure constituting the object of the present invention, differing from the procedure cited in the foregoing, starch is used for substrate and cellobiose and/or trehalose for acceptor. No such production method has been described heretofore. One obtains by this production method a novel, unique oligosaccharide mixture which is usable as a new raw material e.g. in the foodstuff and animal feed industries.

The object of the invention is an oligosaccharide mixture containing trehalose and cellobiose as acceptor sugar, and a procedure for manufacturing same. In the procedure, cyclomaltodextrin-glucanotransferase (CGTase; E.C. 2.4.1.19) is made to act on starch in the presence of acceptor sugar (trehalose or cellobiose), whereby oligosaccharides are formed which in their sugar composition contain trehalose and cellobiose. For the reason mentioned above, we have arrived at the invention as disclosed in the application, which is characterized by the features stated in the claims.

The basic idea of the invention consists in that CGTase has been found to produce oligosaccharides from a suitable acceptor sugar and starch, in suitable conditions. It is possible in the procedure of the invention, by varying the conditions of reaction, to achieve oligosaccharide mixtures of novel type in which the oligosaccharides have DP numbers starting with 3 and up to 7 at least. The reaction model of the procedure for producing an oligosaccharide mixture, on which the invention reads, can be assumed to be as follows,

when cellobiose ( $\beta$ -glu-(1-4)- $\beta$ -glu) or trehalose ( $\alpha$ -glu-(1-1)- $\alpha$ -glu) is used for acceptor.

5                    starch + acceptor    ----                    acceptor  
   glu-acceptor (OSG3)  
   glu-glu-acceptor (OSG4)  
   glu-glu-glu-acceptor (OSG5)  
   etc.

10                    where acceptor = trehalose or cellobiose (disaccharide)

It was found in  $^{13}\text{C}$ -nmr structural analysis that the trehalose-containing oligosaccharide OSG3 has the following structure: O- $\alpha$ -D-glu-(1-4)-O- $\alpha$ -D-glu-(1-1)- $\alpha$ -D-glu, that is, a glucoside molecule is attached to trehalose with an  $\alpha$ -(1-4)-glycosidic bond. The oligosaccharides with DP higher than 3 are hard to analyse by nmr technique, owing to their complex structure. It has, however, been found in enzymatic structural analysis that the glucose molecule(s) are linked with the acceptor sugar in conformity with the above model. Oligosaccharides according to the invention can be used e.g. in the foodstuff industry as new raw materials, because  $\alpha$ -amylase breaks up very slowly, or not at all, oligosaccharides with DP between 3 and 5 (OSG3-OSG5) (e.g. in: Starch, Chemistry and Technology (1984) edited by R.L. Whistler, J.N. BeMiller and E.F. Paschall, Academic Press Inc., London, England, p. 93-102). The oligosaccharide mixture obtained as product may furthermore contain the acceptor sugars trehalose or cellobiose, which are also used as single sugars for raw materials of the foodstuff industry. The proportion in the product mixture of oligosaccharides having DP 3-5 can be modified by means of dry matter and enzyme concentration, relative mass proportion of starch and acceptor sugar, and reaction time.

The compositions of the product mixtures were

determined by liquid chromatography. The method enabled the concentrations of oligosaccharides with DP less than 8 to be determined in that the concentration of any given oligosaccharide was calculated in accordance with the concentration of whichever standard (DP from 1 to 7) had the most closely equal retention time. Confirmation of the results was made qualitatively with TLC.

The CGTase required in the present invention is produced by cultivating a microorganism producing the respective enzyme, e.g. certain bacteria of genus *Bacillus*, in a culture solution containing a carbon and nitrogen source, minerals and vitamins (M. Mäkelä, Biotechnical production of cyclodextrins (1990), Department of Biochemistry, University of Turku, Finland). The CGTase thereby formed is recovered using procedures of prior art, such as centrifuging the culture solution, or filtering. The crude enzyme saved in this manner may be purified and concentrated e.g. by salting out, gel filtration and/or by ion exchange chromatography or affinity chromatography.

In the procedure starch materials of various origins can be used, e.g. those derived from cereals such as barley starch, or from root crops, such as potato starch. The starch may also be pretreated e.g. by acid hydrolysis and/or enzymatically so that the dextrose equivalent of the liquefied starch thus formed is in the range from 0.5 to 20. For enzymatic hydrolysis of starch one may use e.g.  $\alpha$ -amylase or it is also possible to add the production enzyme, or CGTase, directly to the starch, in which case pretreatment is unnecessary. The advantageous DE number of the starch is about 1.

The total concentration of starch reacting with CGTase and acceptor sugar should be within 5-60%. Advantageous concentration is 30-40% for production of cellobiose saccharides, and 40-50% for production of

trehalose saccharides, whereby the relatively greatest amount of short-chain oligosaccharides will be formed in the mixture. Particularly in production of trehalose oligosaccharides the concentration should be high. Substrate and acceptor may be dissolved either in water or in buffer, e.g. in 50 mM imidazole buffer pH 6.8, in 50 mM acetate buffer pH 5.5 or in 50 mM glycine-NaOH buffer pH 9.0. Favourable pH range for the reaction is 6.5-7.0. The reaction may be carried out at 50-80°C, with reaction time 2 days at the most. Favourable temperature for the reaction is 60°C and reaction time, 20 to 48 hrs, depending on the CGTase concentration. The relative mass proportion of starch and acceptor sugar should be within 0.5-4 in the solution. Favourable proportion of starch and acceptor is 1:1, whereby OSG3-OSG5 oligosaccharides will be formed in the mixture most of all. When the starch quantity exceeds that of acceptor, long-chain oligosaccharides are formed most, in proportion. Similarly, when there is more acceptor than starch in the solution, short-chain oligosaccharides, among others the OSG3 oligosaccharide, are formed relatively most. The CGTase concentration in the reaction should be within 30-350 U per g of starch. The favourable CGTase concentration is affected, among others, by concentration in that the higher the concentration of the solution the higher should the enzyme concentration be in the reaction. High enzyme concentration also shortens the reaction time.

It is thus understood that it is possible in the procedure of the invention, by appropriately modifying conditions of reaction, to produce in controlled manner, and with good yield, oligosaccharide mixtures containing cellobiose and trehalose, of a novel type.



EXAMPLE 1Manufacturing cellobiose oligosaccharides at various concentrations

Oligosaccharide-production enzyme, or CGTase, (isolated from a *Bacillus circulans* strain, activity 7600 U/ml, Oy Alko Ab) was added 30 U/g to a mixture containing starch as stated in Table 1, in 50 mM imidazole buffer pH 6.8, to which had been added 1.5 mM  $\text{CaCl}_2$ . The CGTase was allowed to act at 85°C for 30 min. with simultaneous agitation, whereafter in the starch (DE 1) was dissolved cellobiose (Sigma, U.S.A.) as stated in Table 1, and the solution was tempered to 60°C reaction temperature.

The reaction was started by adding to the solution 50 U CGTase per g of starch, and the reaction was allowed to proceed 48 hrs at 60°C, agitating at the same time. The compositions of the products were determined at room temperature by liquid chromatography (Zsardon B., Otta K.H., Tudos F. and Szejtli J. (1979), *J. Chromatogr.*, 172, 490-492). The elution rate in carbohydrate column was 0.9 ml/min and the standards (e.g. glucose, maltose, ..., naltoheptaose, cellobiose, trehalose, cyclodextrin) had concentration from 1 to 5 mg/ml.

The concentrations of OSG3-OSG7 oligosaccharides in the products after 48 hrs reaction time are stated in Table 1. The total quantity of oligosaccharides OSG3-OSG5, at 10% dry matter content, was 4.8 g/100 g (48.0% of the initial dry matter content) and at 30% dry matter content, 14.6 g/100 g (48.7% of initial dry matter content). In Fig. 1 is presented the yield of OSG3-OSG5 oligosaccharides and the cellobiose consumption (g/100 g) plotted over time (48 hrs) at 30% dry matter content.

TABLE I. The effect of substrate solution concentration (g/100 g) on composition of the oligosaccharide mixture produced (Example 1). Starch (DE 1) was reacted with CGTase (50 U/g) for 48 hrs in presence of cellobiose (mass proportion of starch and acceptor in the substrate, 1:1). The concentrations of oligosaccharides (with DP less than 8) were determined by liquid chromatography (see Example 1).

Product	Dry matter content in solution (%)			
	10%	30%	10%	30%
Product	Concentration (g/100 g)			
	Prior to reaction		After reaction	
Starch	5.0	15.0		
Glucose			0.03	0.04
Cellobiose	5.0	15.0	2.1	6.9
OSG3			2.0	6.0
OSG4			1.6	5.0
OSG5			1.2	3.6
OSG6			0.8	2.9
OSG7			0.2	0.9

## EXAMPLE 2

### Manufacturing trehalose oligosaccharides at various concentrations

Oligosaccharide producing enzyme, or CGTase (see Example 1) was added 30 U/g to a mixture containing starch, as stated in Table 2, in buffer as in Example 1. The CGTase was allowed to act for 30 min, at 85°C, with agitation, whereafter in the starch (DE 1) was dissolved  $\alpha,\alpha$ -trehalose (Sigma, U.S.A.) as stated in Table 2, and the solution was tempered to the reaction temperature, 60°C.

The reaction was started by adding CGTase 150 U per g of starch, and the reaction was allowed to proceed 48 hrs at 60°C under agitation. The compositions of the products were determined by liquid chroma-

tography (see Example 1).

The concentrations of OSG3-OSG7 oligosaccharides in the products after 48 hrs reaction time are stated in Table 2. The total quantity of oligosaccharides OSG3-OSG5, at 32% dry matter content, was 12.1 g/100 g (37.8% of the initial dry matter content) and at 50% dry matter content, 20.0 g/100 g (40.0% of initial dry matter content).

TABLE II. The effect of substrate solution concentration (g/100 g) on composition of the oligosaccharide mixture produced (Example 2). Starch (DE 1) was reacted with CGTase (150 U/g) for 48 hrs in presence of trehalose (mass proportion of starch and acceptor in the substrate, 1:1). The concentrations of oligosaccharides (with DP less than 8) were determined by liquid chromatography (see Example 1).

		Dry matter content in solution (%)			
		32%	50%	32%	50%
	Product	Concentration (g/100 g)			
		Prior to reaction		After reaction	
25	Starch	16.0	25.0		
	Glucose			0.2	0.1
	Trehalose	16.0	25.0	8.5	11.5
	OSG3			4.5	9.1
	OSG4			4.8	6.6
30	OSG5			3.5	4.3
	OSG6			2.6	2.7
	OSG7			2.4	1.3

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### EXAMPLE 3

#### Effect of starch/acceptor mass proportion in oligosaccharide production

CGTase (see Example 1) was added 30 U/g to a mixture containing starch in the mass proportion stated in Table 3, so that the ultimate concentration after

40

acceptor addition in the solution would be 30 g/100 g. The starch had been dissolved in imidazole buffer as in Example 1. The CGTase was allowed to act for 30 min, at 85°C, with agitation, whereafter in the starch (DE 1) 5 was dissolved either trehalose or cellobiose as stated in Table 3 (ultimate concentration 30 g/100 g), and the solution was tempered to 60°C reaction temperature.

The reaction was started by adding CGTase 50 U per g of starch, and the reaction was allowed to proceed 10 48 hrs at 60°C under agitation. The compositions of the products were determined by liquid chromatography (see Example 1).

The concentrations of OSG3-OSG7 oligosaccharides in the products after 48 hrs reaction time are 15 stated in Table 3. The total quantities of oligosaccharides OSG3-OSG5 were, with the cellobiose saccharides, 13.1 g/100 g (mass proportion 1:4) and 14.4 g/100 g (1:2), and with trehalose saccharides 6.9 g/100 g in either case (mass proportions 1:2.3 and 1:1.2). The 20 proportion of the OSG3 oligosaccharide was highest when the acceptor concentration in the solution was high.

TABLE III. The effect of starch/acceptor mass proportion on composition of the oligosaccharide mixture produced (Example 3). Starch (DE 1) was reacted with CGTase (50 U/g) for 48 hrs in presence of cellobiose or trehalose at 30% concentration. The concentrations of oligosaccharides (with DP less than 8) were determined by liquid chromatography (see Example 1).

Starch/acceptor mass proportion	Cellobiose		Trehalose(%)	
	1:4	1:2	1:2.3	1:1.2
Product	Concentration (g/100 g)			
Starch	0.4	0.03	0.08	0.09
Cellobiose	18.9	13.0	-	-
Trehalose	-	-	17.4	13.9
OSG3	8.4	7.8	2.7	3.3
OSG4	3.4	4.2	2.4	2.3
OSG5	1.3	2.4	1.8	2.4
OSG6	+	1.6	1.3	2.2
OSG7	0.4	0.3	0.9	1.6

#### EXAMPLE 4

#### Manufacturing trehalose oligosaccharides at various enzyme concentrations

Trehalose oligosaccharides were produced in the manner described in Example 2, in the reaction being used 50 g starch (DE 1) and 50 g trehalose dissolved in 200 g imidazole buffer (dry matter content 50%). The reaction was started by adding CGTase (see Example 1) 150 and 210 U per g of starch, and the reaction temperature was 60°C.

The composition of the product was determined by liquid chromatography (see Example 1). In Fig. 2 the yield of oligosaccharides and the consumption of trehalose (g/100 g) are plotted over time (48 hrs, CGTase concentration 210 U/g). Fig. 3 displays the elution chromatogram from liquid chromatography of the oligo-

saccharide mixture after 48 hrs reaction time. This chromatogram represents a typical oligosaccharide mixture, its numbered peaks being: 1=glucose, 2=trehalose, 3=OSG3, 4=OSG4, 5=OSG5, 6=OSG6, and 7=OSG7.

5           The concentrations of OSG3-OSG5 oligosaccharides after 48 hrs reaction time are presented in Table 4. The total of OSG3-OSG5 oligosaccharides in the product was measured to be 20.0 g/100 g (40.0% of the dry matter content, CGTase 150 U/g) and 22.7 g/100 g (45.4%  
10 of the dry matter content, CGTase 210 U/g).

TABLE IV. The effect of enzyme concentration on composition of the oligosaccharide mixture produced (Example 3). Starch (DE 1) was reacted with CGTase for  
15 48 hrs in presence of trehalose at (mass proportion of starch and acceptor in the substrate 1:1, dry matter content 30%). The concentrations of oligosaccharides (with DP less than 8) were determined by liquid chromatography (see Example 1).

	Enzyme concentration (U/g)	
	150	210
Product	Concentration (g/100 g)	
25 Glucose	0.1	0.2
Trehalose	11.5	10.3
OSG3	9.1	9.8
OSG4	6.6	7.4
30 OSG5	4.3	5.5
OSG6	2.7	3.5
OSG7	1.3	1.6

#### 35 EXAMPLE 5

##### Hydrolyzability with amylase of cellobiose oligosaccharides

The production solution of Example 1, with 30% dry matter content, was diluted with water to 4.72% dry  
40 matter content and centrifuged, to remove the dry matter. To the solution was added 135.5 U/ml  $\alpha$ -amylase

(isolated from a *Bacillus subtilis* strain, activity 1355 U/mg, Sigma, U.S.A.) and the reaction was allowed to proceed 24 hrs at 60°C, with agitation. The composition of the product was determined by liquid chromatography (see Example 1), and measurement showed the total content of OSG3-OSG5 oligosaccharides in the product to be 14.2 g/100 g (OSG3: 7.3 OSG4: 4.2 g/100 g, OSG5: 2.7 g/100 g).

$\alpha$ -amylase broke off 16% oligosaccharide and 25% OSG5 oligosaccharide from the product of Example 1, while on the other hand the content of OSG3 oligosaccharide increased 22%.

#### EXAMPLE 6

##### 15 Manufacturing of trehalose oligosaccharides and their hydrolyzability with amylase

Trehalose oligosaccharides were produced in the manner described in Example 2, in the reaction being used 15 g starch (DE 1) and 13.6 g trehalose dissolved in 200 g imidazole buffer (dry matter content 28.6%). The reaction was started by adding CGTase (see Example 1) 50 U per g of starch, and the reaction temperature was 60°C. The reaction was allowed to proceed for 48 hrs, whereafter the composition of the product was determined by liquid chromatography (see Example 1). Measurement showed the total quantity of OSG3-OSG5 oligosaccharides to be 6.6 g/100 g (OSG3: 1.9 g/100 g, OSG4: 2.3 g/100 g, OSG5: 2.4 g/100 g, total 23.1% of the dry matter content).

The production solution was diluted with water to 6% dry matter content and centrifuged, to remove the dry matter. To the solution was added 271 U/ml  $\alpha$ -amylase (see Example 5) and the reaction was allowed to proceed 24 hrs at 60°C, with agitation. The composition of the product was determined by liquid chromatography (see Example 1), and measurement showed the total quantity of OSG3-OSG5 oligosaccharides in the product to be

10.7 g/100 g (OSG3: 5.8 OSG4: 3.3 g/100 g, OSG5: 1.6 g/100 g).  $\alpha$ -amylase broke off 33% OSG5 oligosaccharide from the product, while on the other hand the content of OSG3 oligosaccharide was tripled.

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#### EXAMPLE 7

##### Use of starches with various DE numbers in manufacturing oligosaccharides

Trehalose oligosaccharides were produced in the manner described in Example 2, but the starch was first hydrolyzed with  $\alpha$ -amylase (BAN 120 L, activity 120 KNU/g, Novo, Denmark). Amylase was added 0.3 and 0.9 KNU/g to a mixture containing 16 g starch in imidazole buffer (see Example 2). The amylase was allowed to act 30 min. at 85°C, with stirring, whereafter in the starch (DE 5 and DE 16) was dissolved 16 g trehalose (dry matter content of solution 32%) and the solution was tempered to reaction temperature, 60°C.

The reaction was started by adding to the solution CGTase (see Example 1) 120 U per g of starch, and the reaction was allowed to proceed 48 hrs at 60°C, with stirring. The composition of the products was determined by liquid chromatography (see Example 1) and by TLC on silicagel plates (running solution: acetone-1-butanol-water 11:9:5; staining solution: aniline 2 ml, diphenylamine 2 g, acetone 100 ml, and 80% phosphoric acid 15 ml; after plate staining, 30 min. heating at 105°C).

The total quantity of OSG3-OSG5 oligosaccharides in the product was found, in either case, to be 11.5 which was 35.9% of the dry matter content. The product compositions were also analysed by TLC, which differentiates the produced oligosaccharides from the hydrolysis products formed in the pretreatment of the starch, such as maltose, maltotriose, maltotetraose, etc. According to TLC the product solutions contained very small quantities of hydrolysis products formed in



the pretreatment; their concentrations varied within 0.1 to 2.5 g/100 g, depending on the DE number of the solution.

Using starch with DE 5, the oligosaccharide contents of the product were found by measurement to be: OSG3: 4.4 g/100 g, OSG4: 3.9 g/100 g, OSG5: 3.2 g/100 g, and with starch having DE 16: OSG3: 4.6 g/100 g, OSG4: 4.0 g/100 g, OSG5: 2.9 g/100 g.

#### 10 EXAMPLE 8

##### Use of various CGTase preparations and buffers in manufacturing trehalose oligosaccharides

CGTase (see Example 1) was added 30 U/g to a mixture containing 2 g starch in water or in 50 mM acetate, imidazole or glycine-NaOH buffer, to which had been added 1.5 mM  $\text{CaCl}_2$ . The buffers had pH 5.5, 6.8 and 9.0, respectively. CGTase was allowed to act 30 min, at 85°C, with stirring, whereafter in the starch (DE 1) was dissolved 3 g trehalose (dry matter content of solution 40%) and the solution was tempered to reaction temperature 60°C.

To the reaction mixtures was added, for production enzyme, either purified CGTase (see Example 1, activity 7600 or 6150 U/ml) or equivalent crude preparation (activity 4300 U/ml), 300 U per g of starch. The reaction was allowed to proceed 24 hrs at 60°C, with stirring. The compositions of the products were determined by liquid chromatography (see Example 1) and are presented in Table V.

- TABLE V. Preparation of trehalose oligosaccharides using various CGTase preparations and buffers at 40% dry matter content. Starch (DE 1) was allowed to react with CGTase (300 U/g) during 24 hrs in the presence of trehalose (starch/trehalose mass proportion 1:1). The oligosaccharide concentrations were determined by liquid chromatography (see Example 1). The yields of oligosaccharides OSG3-OSG5 have been calculated relative to dry matter content.
- Activities of CGTase preparations: A=7600 U/ml, B=6150 U/ml, and C=4300 U/ml.
- Test 1. Acetate buffer pH 5.5, CGTase A.  
 Test 2. Acetate buffer pH 5.5, CGTase C.  
 Test 3. Glycine-NaOH buffer pH 9.9, CGTase C,  
 Test 4. Water pH 7.0, CGTase B.

		Concentration (g/100 g)				
	Product	Test 1	2	3	4	5
20	Glucose	0.2	0.3	0.2	0.1	0.1
	Trehalose	10.9	11.1	10.2	9.0	0.3
	OSG3	7.3	8.8	7.9	7.7	6.5
	OSG4	6.2	6.3	6.5	6.2	5.4
	OSG5	4.8	3.9	4.7	4.4	4.0
25	OSG6	3.2	2.4	3.1	3.0	2.8
	OSG7	2.3	1.1	2.0	2.0	1,7
OSG3-OSG5 yield (%)						
30		45.8	47.5	47.8	45.8	39.8

#### EXAMPLE 9

#### 35 Production of trehalose oligosaccharides with immobilized CGTase

CGTase was bonded with covalent bonds to Eupergit C beads (Röhm Pharma, Federal Republic of Germany) in that to 0.5 g of the beads (dry matter mass) was added 2 ml CGTase (isolated from *Bacillus circulans* strain, activity 265 U/ml, Oy Alko Ab). The enzyme was left to be bonded for 20 hours at room temperature, shaking at the same time, whereafter the

beads were washed with imidazole buffer according to Example 2.

In order to prepare the substrate for the oligosaccharide producing reaction, soluble CGTase was added 30 U/g to a mixture containing 0.5 g starch and 4 g buffer as in Example 1. The CGTase was allowed to act 30 min. at 85°C, with stirring, whereafter in the starch (DE 1) was dissolved 0.5 g trehalose (dry matter content of solution 20%), and the solution was tempered to reaction temperature 60°C.

The reaction was started by adding to the solution immobilized CGTase 0.5 g (dry matter mass, activity 424 U/g), and the reaction was allowed to proceed 21 hrs at 60°C, shaking at the same time. The composition of the product was determined by liquid chromatography (see Example 2). Measurements gave for the total OSG3-OSG5 oligosaccharide quantity in the product: 7.5 g/100 g (OSG3: 3.2 OSG4 2.4 g/100 g, and OSG5: 1.9 g/100 g), which is 37.5% of the dry matter content.

## CLAIMS

1. A procedure for manufacturing an oligosaccharide mixture, characterized in that  
5 cyclomaltodextrin-glucanotransferase (CGTase; E.C. 2.4.1.19) is made to act on starch in the presence of acceptor sugars trehalose and/or cellobiose.

2. Procedure according to claim 1, characterized in that CGTase is made to act on  
10 starch which has been pre-treated.

3. Procedure according to claim 1, characterized in that the CGTase is immobilized CGTase.

4. Procedure according to claim 1, characterized in that the dextrose equivalent of  
15 the starch is within the range 0.5-20.

5. Procedure according to claim 1, characterized in that the dry matter content of the starch and acceptor sugar is within 5-60%, and that  
20 the starch is dissolved either in water or in a buffer having pH in the range 5.5-9.0.

6. Procedure according to claim 1, characterized in that the reaction is carried out at a temperature between 50 and 80°C, that the mass  
25 proportion of starch and acceptor in the solution is in the range 0.5-4, and that the CGTase concentration is in the range 30-350 U per g of starch.

7. A starch-based oligosaccharide mixture manufactured by a procedure according to any one of  
30 claims 1-6, characterized in that it contains glucose 0-5.0%, acceptor sugar 7.0-75%, OSG3 1.8-32%, OSG4 3.0-20%, OSG5 2.5-16%, OSG6 0.8-12% and OSG7 0.2-10%, calculated on the dry matter of the solution, the acceptor sugar being trehalose and/or cellobiose and the oligosaccharides containing trehalose  
35 and/or cellobiose.

8. A starch-based oligosaccharide mixture

according to claim 7, characterized in that it contains glucose 0-1.0 g/100 g, cellobiose 0.7-35 g/100 g, OSG3 0.9-15 g/100 g, OSG4 0,8-9.0 g/100 g, OSG5 0.8-6.0 g/100 g, OSG6 0.2-5.0 g/100 g and 0.05-5 3.0 g/100 g, the oligosaccharides containing cellobiose.

9. A starch-based oligosaccharide mixture according to claim 7, characterized in that it contains glucose 0-2.0 g/100 g, trehalose 7.0-10 35 g/100 g, OSG3 0.4-14 g/100 g, OSG4 0,8-10.0 g/100 g, OSG5 0.8-7.0 g/100 g, OSG6 0.8-6.0 g/100 g and 0.5-5.0 g/100 g, the oligosaccharides containing trehalose.

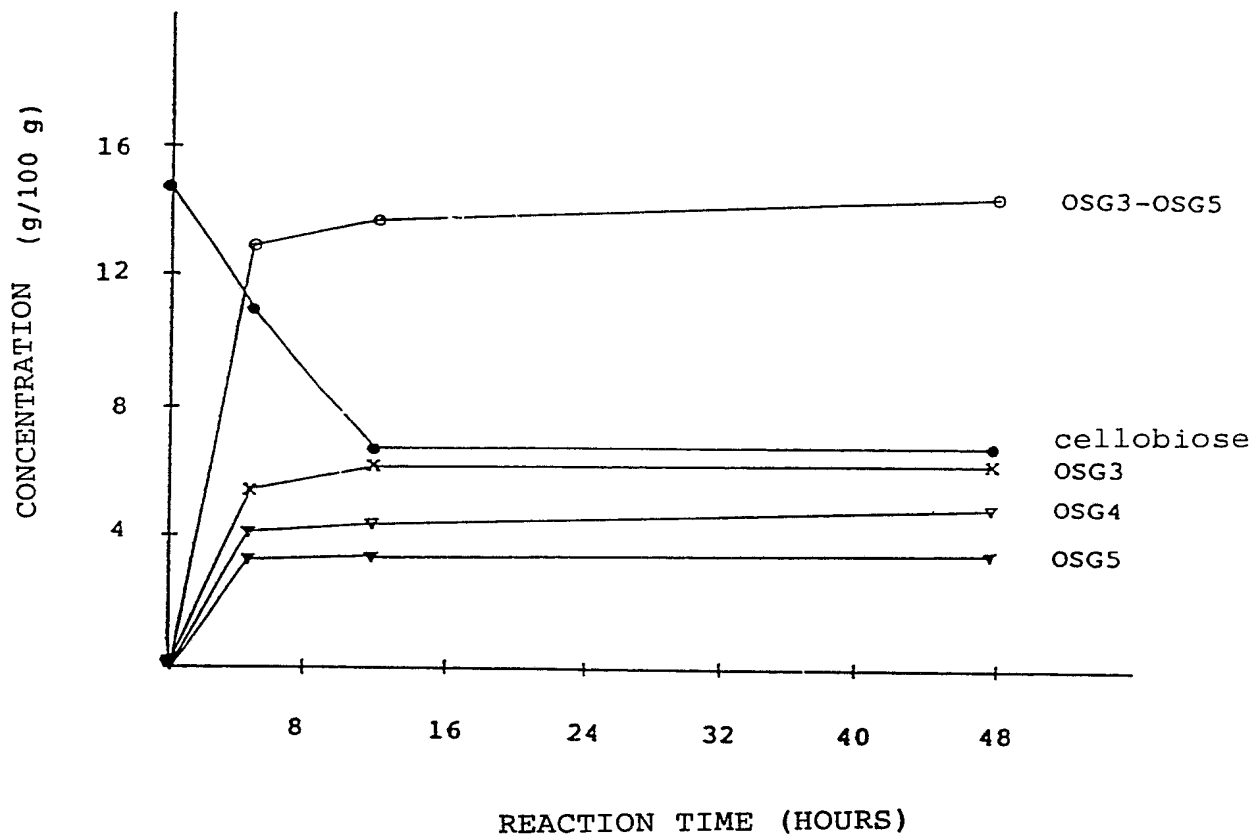


FIG. 1

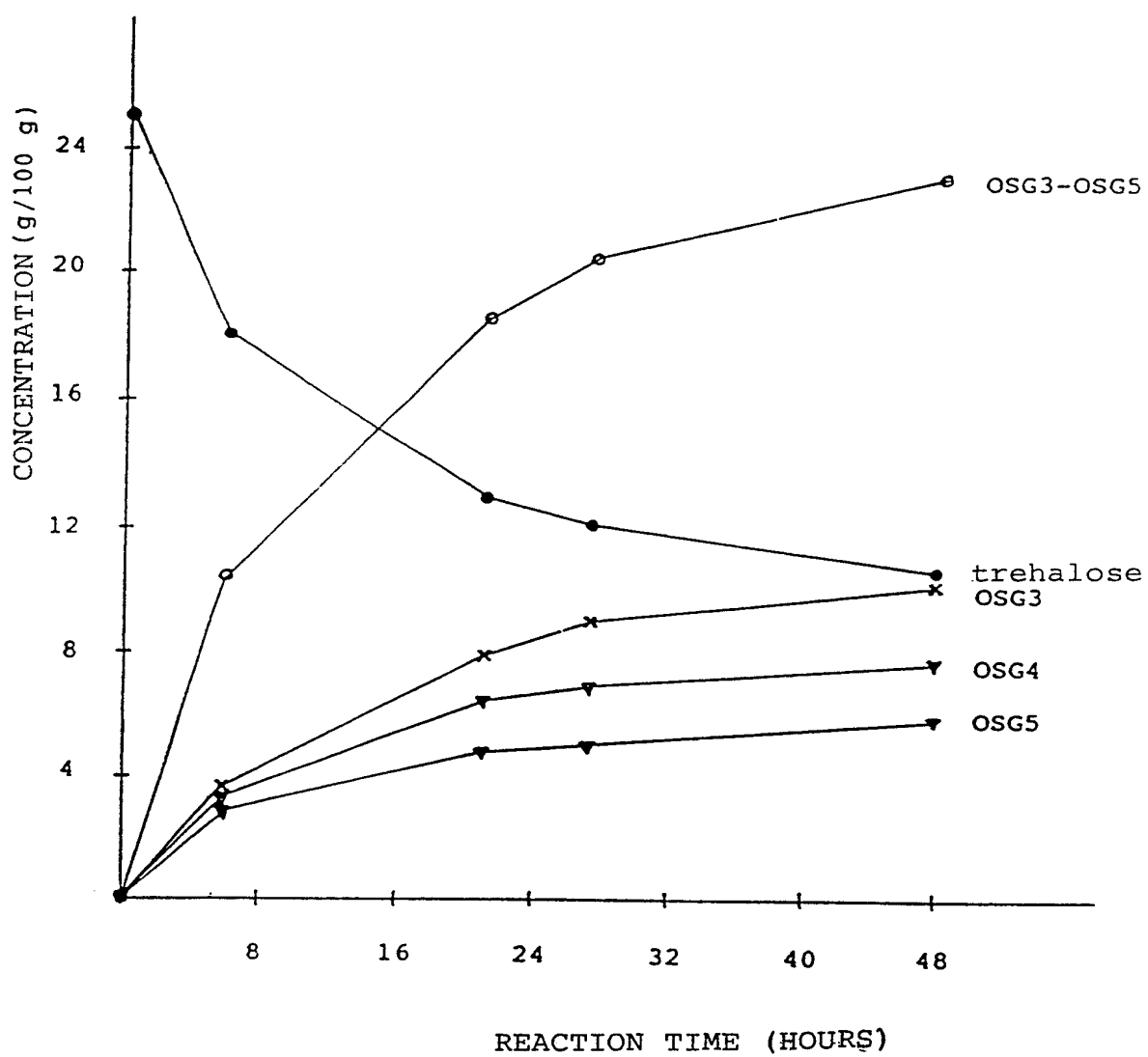


FIG. 2

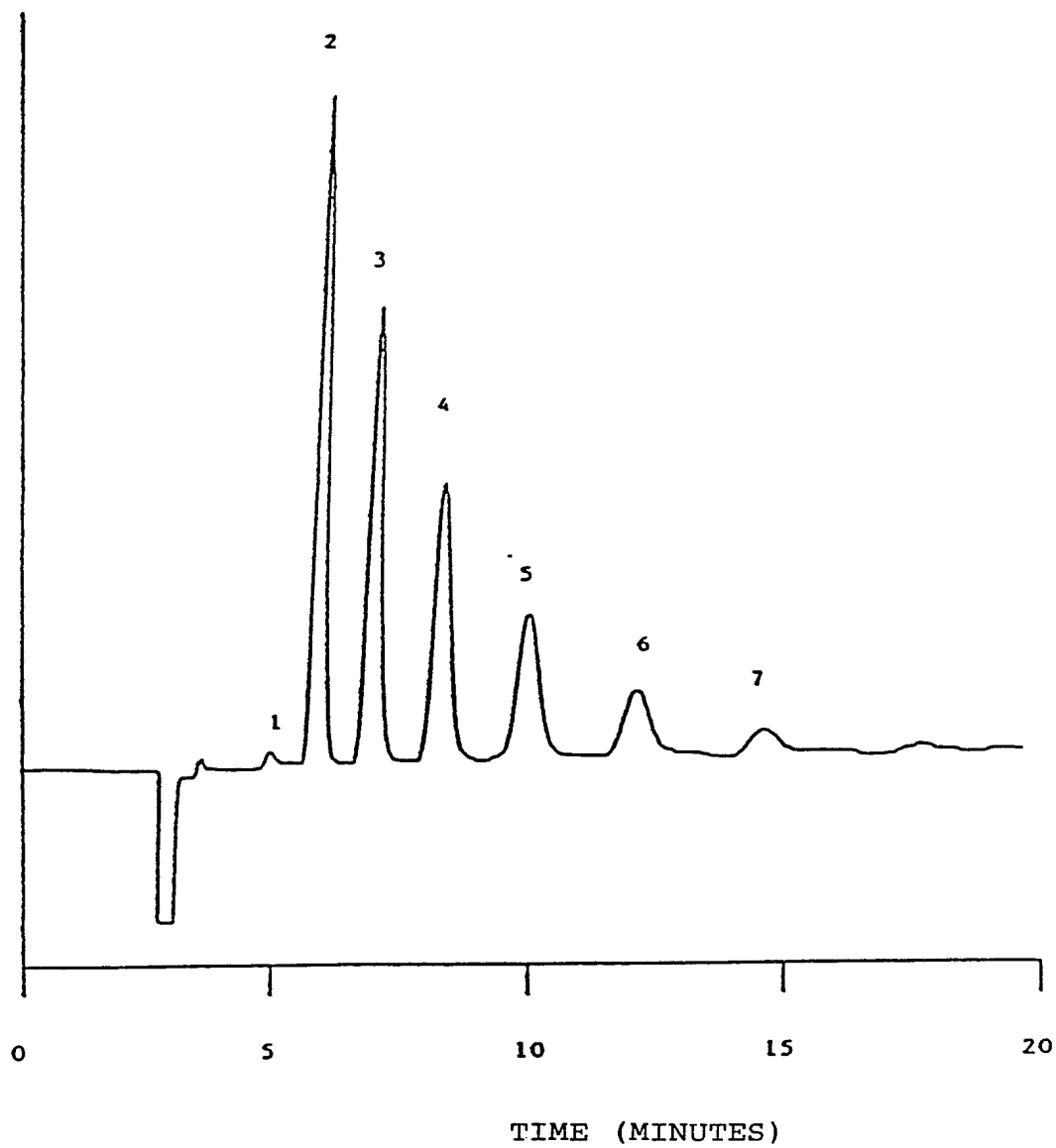


FIG. 3



# INTERNATIONAL SEARCH REPORT

International Application No PCT/FI 91/00239

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC <b>IPC5: C 12 P 19/18</b>														
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched<sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 25%; border-bottom: 1px solid black;">Classification System</td> <td style="border-bottom: 1px solid black;">Classification Symbols</td> </tr> <tr> <td style="height: 40px; vertical-align: bottom;">IPC5</td> <td style="height: 40px; vertical-align: bottom;">C 12 P</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched<sup>8</sup></div> <p style="padding: 5px;">SE,DK,FI,NO classes as above</p>			Classification System	Classification Symbols	IPC5	C 12 P								
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IPC5	C 12 P													
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b> <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%;">Category *</th> <th style="width: 70%;">Citation of Document,<sup>11</sup> with indication, where appropriate, of the relevant passages<sup>12</sup></th> <th style="width: 20%;">Relevant to Claim No.<sup>13</sup></th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>US, A, 4477568 (HENDRIK HOKSE ET AL.) 16 October 1984, see column 1, line 35 - line 38; claim 1 --</td> <td style="text-align: center; vertical-align: top;">1-9</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>DE, A, 2162276 (HAYASHIBARA, KEN) 6 July 1972, see page 2, line 21 - line 30 --</td> <td style="text-align: center; vertical-align: top;">1-9</td> </tr> <tr> <td style="text-align: center; vertical-align: top;">X</td> <td>Chemical Abstracts, volume 104, no. 13, 31 March 1986, (Columbus, Ohio, US), Nakamura, Sanehisa et al.: "Preparation of syrup of sucrose-containing oligosaccharides ", see page 574, abstract 107812j, &amp; Ryukyu Daigaku Nogakubu Gakujutsu Hokoku 1984, 31, 43- 50 --</td> <td style="text-align: center; vertical-align: top;">1-9</td> </tr> </tbody> </table>			Category *	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	US, A, 4477568 (HENDRIK HOKSE ET AL.) 16 October 1984, see column 1, line 35 - line 38; claim 1 --	1-9	X	DE, A, 2162276 (HAYASHIBARA, KEN) 6 July 1972, see page 2, line 21 - line 30 --	1-9	X	Chemical Abstracts, volume 104, no. 13, 31 March 1986, (Columbus, Ohio, US), Nakamura, Sanehisa et al.: "Preparation of syrup of sucrose-containing oligosaccharides ", see page 574, abstract 107812j, & Ryukyu Daigaku Nogakubu Gakujutsu Hokoku 1984, 31, 43- 50 --	1-9
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><b>* Special categories of cited documents:<sup>10</sup></b></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="height: 40px; vertical-align: bottom;">22nd November 1991</td> <td style="height: 40px; vertical-align: bottom;">1991 -11- 28</td> </tr> <tr> <td style="border-bottom: 1px solid black;">International Searching Authority</td> <td style="border-bottom: 1px solid black;">Signature of Authorized Officer</td> </tr> <tr> <td style="height: 40px; vertical-align: bottom; text-align: center;">SWEDISH PATENT OFFICE</td> <td style="height: 40px; vertical-align: bottom; text-align: center;">            Yvonne Siösteen         </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	22nd November 1991	1991 -11- 28	International Searching Authority	Signature of Authorized Officer	SWEDISH PATENT OFFICE	Yvonne Siösteen				
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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 113, no. 19, 5 November 1990, (Columbus, Ohio, US), Kitahata, Sumio: "Synthesis of oligosaccharides by use of microbial enzymes ", see page 567, abstract 170336h, & Denpun Kagaku 1990, 37( 2), 59- 67 --	1-9
A	Chemical Abstracts, volume 112, no. 7, 12 February 1990, (Columbus, Ohio, US), see page 591, abstract 53775u, & JP, A, 1179698 (Manufacture of malto-oligosucroses from starches and sucrose with cyclodextrin glucanotransferase using ultrafiltration membranes) 17 July 1989 --	1-9
A	US, A, 4254227 (SHIGETAKA OKADA ET AL.) 3 March 1981, see the whole document -- -----	1-9

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.PCT/FI 91/00239**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the Swedish Patent Office EDP file on **91-10-31**  
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		JP-B- 58019276	83-04-16